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On the Coupling Between Surface Charge and Hydration in Biomembranes: Experiments with 3-Hydroxyflavone Probes

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The newly synthesized 3-hydroxyflavone derivative [2-(4′-N,N-diethylaminophenyl)-3-hydroxy-6-chromonyl](N,N-dimethyl-octyl) ammonium bromide (F2) together with already used 4′-dimethylamino-3-HF (F) are found to be extremely sensitive to the effects of preferential hydration in model solvent system. This property is explored in the study of phospholipid vesicles of different composition made of neutral, cationic, and anionic lipids. We observe an extremely high level of response of both F and F2 fluorescence emission spectra to the surface charge of the vesicles: the N* form is strongly favored with less positively charged and more negatively charged membrane surface. The strong red-edge effects, which are almost independent of the lipid composition demonstrate the immobility of the probe environment on the time scale of fluorescence emission and suggest the static nature of hydration effects.

KEY WORDS: Membrane surface charge; membrane hydration; fluorescent ratiometric probes; 3-hydroxyflavones.

INTRODUCTION

Hydration of the phospholipid bilayer surface is an important property of biomembranes. The water molecules can penetrate deeply into the bilayer and have hydration effects on the level of phosphate and carbonyl groups, which are easily detected by IR and Raman spectroscopic studies [1–3]. These results are supported by molecular dynamics simulations [4,5]. The estimated hydration level is four water molecules per phosphate group and one per carbonyl group [1]. Both experiments and simulations are in line with the conclusion that hydration depends upon the packing density and dynamics of phospholipid heads. Hydration is expected to be higher when the repulsive forces between negatively charged phosphate groups are not counter-balanced by positive charges of the lipid heads. Indeed the studies of C=O stretching bands of two phospholipid ester carbonyl groups by IR and Raman spectroscopy [2,3] demonstrated higher hydration of these groups in phosphatidylglycerol (PG), which contains only the negative charge on phosphate group, compared to zwitterionic phosphatidylcholine (PC). It is apparent that the important role of water molecules is not only to provide plastification of the bilayer, but also to stabilize dielectrically the dipoles of lipid heads and thus produce a dramatic modulation of the interfacial dipolar potential [6–8]. Meanwhile, the coupling of lipid hydration and formation of dipolar potential is very poorly understood, and the frequently occurring debates [9–11] are based on a very limited amount of evidence.

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This lack of evidence partly results from the limitations of the experimental methods based on fluorescence and especially of the dyes actually available to study the effects of lipid charge on the bilayer hydration. The charged fluorescence probes (e.g., ANS [12,13]) cannot protrude deeply into the bilayer, while the neutral ones (e.g., Laurdan or Prodan [14–16]) are not sensitive enough to the effects of charge. The NBD-based neutral probe was reported to respond to electrostatic membrane potential, but by variation of fluorescence intensity only [17]. The interfacial dipole potential can be probed by the shifts in excitation and emission spectra of electrochromic styryl dyes, but these shifts are small in magnitude and not very sensitive to lipid composition [6–8]. The fluorescence emission decay of DPH is sensitive to bilayer hydration [18], but the reports on the studies of charge effects with this probe are lacking. Therefore new approaches and new probes are in a great demand.

3-Hydroxyflavones (3-HF) have recently attracted the attention of researchers as promising candidates for the design of highly sensitive fluorescence probes for biomembrane research [19,20]. Their sensitivity is based on ratiometric response to different perturbations, which is easily detected in fluorescence spectra. These molecules exhibit an excited-state intramolecular proton transfer (ESIPT) reaction and consequently a dual fluorescence with two emission bands, belonging to the initially excited normal (N*) and reaction product tautomer (T*) forms. Both of these forms can provide highly emissive fluorescence bands that are well-separated on the wavelength scale [21–24]. The ratio of intensities of N* and T* bands can be a very sensitive parameter that describes the probe response.

In the present report we provide the results of comparative studies of vesicles composed of phospholipids containing different charges. In these studies we apply the newly synthesized 3-HF derivative [2-(4′-N,N-diethylaminophenyl)-3-hydroxy-6-chromonyl](N,N-dimethyloctyl) ammonium bromide (F2) together with already used [24] 4′-dimethylamino-3-HF (F). Addition of quaternary ammonium group together with hydrophobic octyl chain is expected to provide the flavone molecule with more determined location in lipid bilayer. Furthermore, F2 in PC vesicles shows N* and T* bands of almost the same intensities. In this study we demonstrate primarily the dramatic sensitivity of these probes to the presence of small amounts of water in aprotic environments. In the next step we apply this property to study the connection between hydration and the charge properties of biomembrane surfaces.

MATERIALS AND METHODS

Flavone F was synthesized and purified as described elsewhere [24]. The synthesis and purification of flavone F2 will be published separately. The chemical structures are presented in Fig. 1. The purity was proved with ^{1}HMR, mass spectrometry, and microanalysis.

Titration of ethyl acetate (of spectroscopic quality from Aldrich) with water was performed by addition of 4 μL of water aliquots to 2 ml of $5 \cdot 10^{-6}$ M solutions of F or F2 in ethyl acetate, to increase gradually the concentration of water by 0.11 M increments up to final concentration 1.1 M (2%, v/v). Large unilamellar vesicles (0.11–0.12 μM in diameter) were obtained by extrusion on polycarbonate filters as previously described [25]. They were made of egg yolk phosphatidylcholine (EYPC) and/or phosphatidylglycerol (EYPG), both from Sigma, and of the cationic lipid DMTAP, a gift from Dr. Heissler (Strasbourg). Absorption spectra were performed on Cary 3 Bio (Varian) spectrophotometer. Fluorescence spectra in solvents were recorded on Quanta Master (PTI) and those in vesicles on SLM 48000 (SLM-Aminco) spectrofluorometers.

RESULTS

In the present study we started from the known fact that fluorescence spectra of flavone F and its analogs are extremely sensitive to the properties of solvent environment [15,16,18]. The increase of polarity and hydrogen bonding ability of the solvent led to dramatic increase of N* relatively to T* form, which is due to a greater dielectric stabilization of N* form. In phospholipid membranes the probe spectra resemble low-polar aprotic solvents with polarity between ethyl acetate and acetonitrile (to be published). Because the fluorophore is neutral and its environment is low-polar, the variation of intensities between N* and T* forms can be the result of the phospholipid-charge–dependent differences in the access of probe to water molecules in the bilayer. We decided to evaluate the magnitude of these effects in simple binary solvent systems.

Fluorescence spectra of probes F and F2 were measured in ethyl acetate on the addition of small amounts of water (Fig. 2). With the increase of water concentration for both flavones we observe a very strong, even dramatic, increase of fluorescence intensity of N* form. Initially in ethyl acetate only a small amount of N* form is observed in emission of probe F. But when water concentration is increased up to 1.1 M (2% by volume), the N* and T* forms become of nearly the same intensity. The

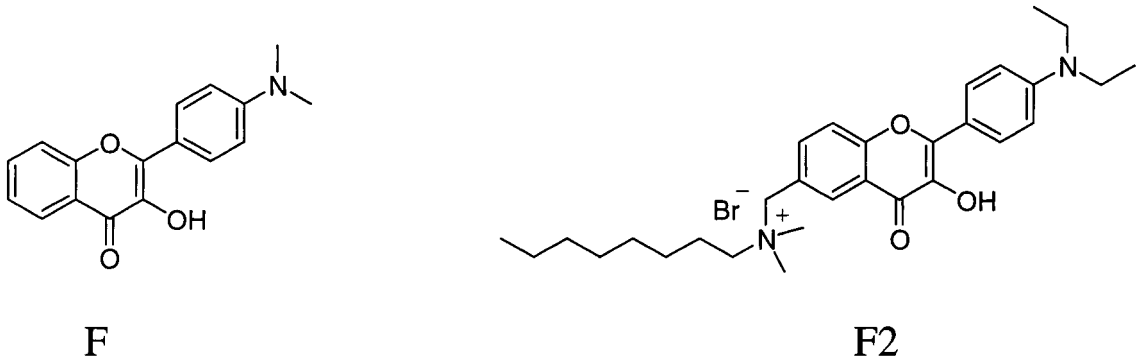

Fig. 1. Chemical structures of 3-hydroxyflavones derivatives.

ratio of N* and T* band intensities increases 4.3 times. Simultaneously, a gradual shift of the N* band maximum to longer wavelengths is observed. For the case of flavone F2, the N* band being minor in neat ethyl acetate becomes the major band with the very small contribution of T* band with the addition of 1.1 M of water. Thus, the observed results demonstrate a possibility of strong connection between fluorescence properties of probes F and F2 and their hydration in low-polar environments.

When incorporated into phospholipid vesicles, F and F2 show two fluorescence bands (Fig. 3). The differences detected in model experiment between these two probes in the ratio of N* and T* forms (see Fig. 2) are also apparent here, but are much smaller. In model solvents the efficiency of ESIPT emission of F is higher than that of F2 by approximately 8, 3, and 2 times in toluene, ethyl acetate, and ethanol, respectively, while in PC vesicles

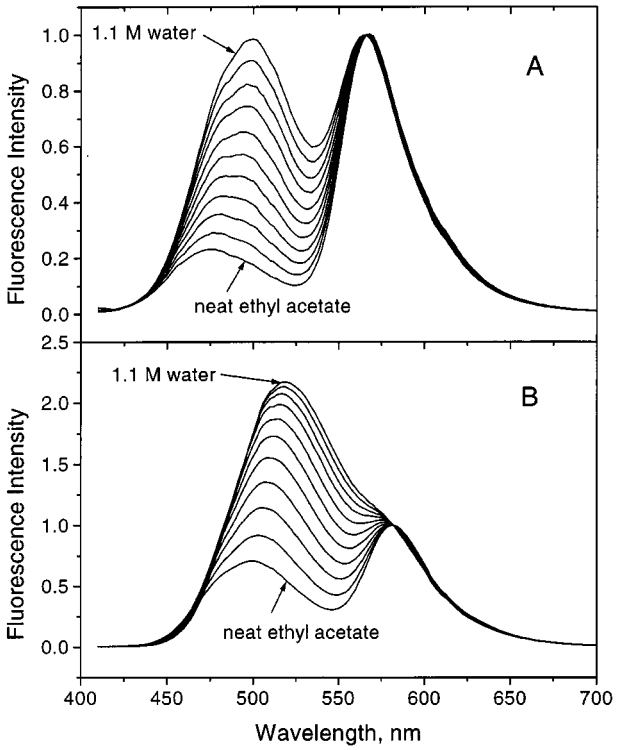

Fig. 2. The effects of addition of water to ethyl acetate on fluorescence spectra of probes F (A) and F2 (B). Each addition of water was 0.11 M (0.2%, v/v). Probe concentration $5 \times 10;^{-6}$ M. The spectra are normalized at long-wavelength maximum.

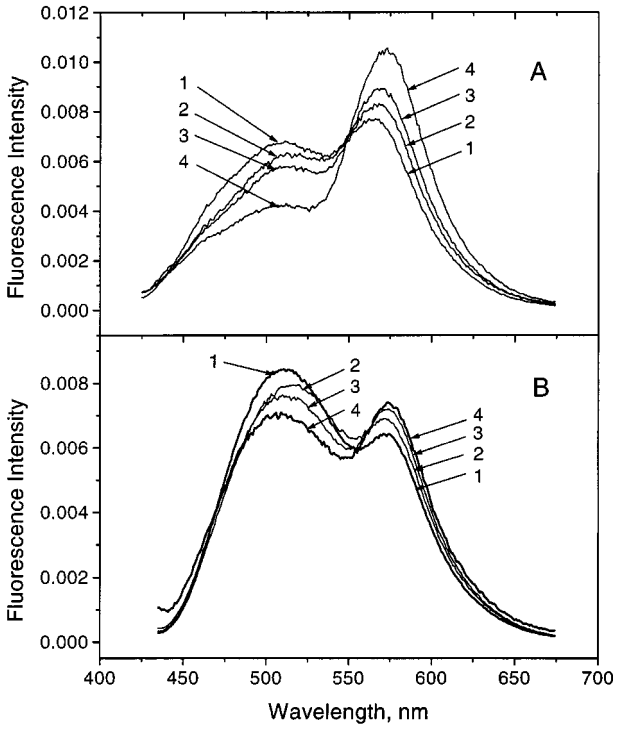

Fig. 3. Fluorescence spectra of flavones F(A) and F2 (B) in large unilamellar vesicles of different composition. Vesicles are composed of: EYPG (1), EYPG/EYPC, 1:1, mol/mol (2), EYPC (3) and DMTAP/EYPC, 1:1, mol/mol (4). The spectra are normalized by integral intensity. Excitation wavelengths: 400 nm (flavone F) and 420 nm (flavone F2). Lipid to probe ratio: 100 mol/mol. Buffer: Hepes 15 m*M*, pH 7.4.

this difference is only by 1.3 times. This can be due to compensation of electrochromic effect (intrinsic Stark effect) of a positively charged ammonium group [26] with negatively charged phosphates of phospholipids. This is also in line with the fact that in PC vesicles the position of N* band is almost the same for both the probes, while in the model solvents, flavone F2 shows a significantly red-shifted N* band compared with that of F.

When the probes F and F2 are incorporated into large unilamellar vesicles of different composition with a strong variation of surface charge, their fluorescence spectra exhibit dramatic variations. For probe F the decrease of negative charge and increase of positive charge per lipid molecule from −1 (for PG) to +0.5 (for DMTAP/PC mixture) results in sequential decrease up to 2.2 times the contribution of N* band relative to T* band. We observed the same effect but somewhat smaller in amplitude (up to 1.4 times) for F2.

The correspondent excitation spectra recorded for different lipids and lipid mixtures at N* and T* fluorescence band maxima (not shown) superimpose totally (with the exception in the case of PG of almost negligible additional contribution to intensity at long-wavelength edge). Thus, for all studied cases, both N* and T* emission bands originate from the same ground-state species, as it should be for the excited-state reaction. Because the variations of excitation spectra in the vesicles of different composition are not detected, the probes should be located in the environments of approximately similar polarity.

The shift of fluorescence spectra at the change of excitation wavelength (which is commonly known as the red-edge effect) is an indicator of immobility of the fluorophore environment on the time scale of emission [27–29]. In membranes it is also an indicator of penetration depth of the probe: this effect is not observed for the charged probes ANS and TNS located at the interface [30] but is apparent for neutral phenylnaphthylamine, which is located deeper in the bilayer [31]. The observation of this effect with flavone probe has recently been reported for DMPC and DPPC vesicles [20]. In the present study we observe strong red-edge effects for both F and F2 probes in all types of studied vesicles. These results are illustrated in Fig. 4. The shift of excitation wavelength from the band maximum to its long-wavelength edge not only produces the shift of the N* band, but also increases its relative intensity over the T* band up to its almost complete disappearance in the case of probe F in EYPG. These effects are accompanied by a substantial decrease of the width of the N* band. Altogether these results demonstrate a rather deep location of probes F and F2 in the bilayers and of the immobility of their surrounding on the nanosecond time scale.

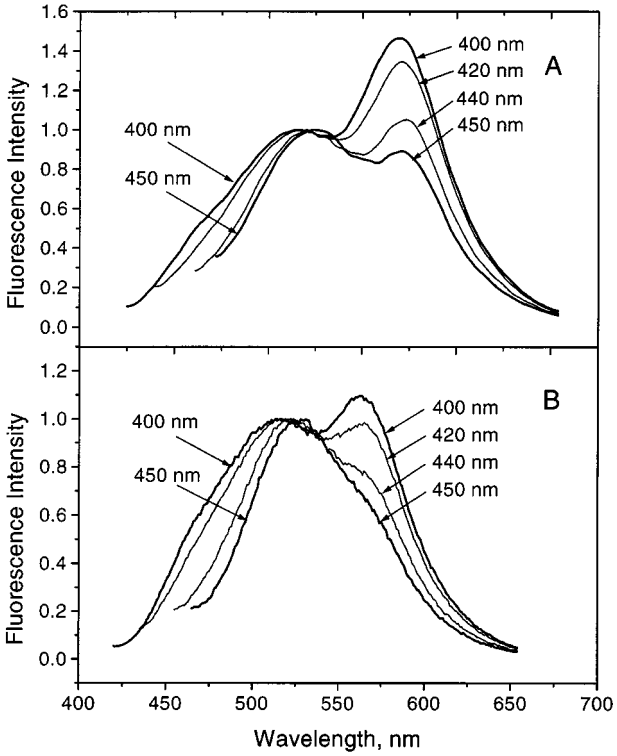

Fig. 4. Dependence of fluorescence spectra of probe F in EYPC (A) and EYPG (B) vesicles on excitation wavelength. The spectra are normalized at short-wavelength maxima. Experimental conditions are the same as in Fig. 3.

DISCUSSION

Thus, the studied probes demonstrate an exceptional sensitivity to phospholipid composition and particularly to the charge of the bilayer surface, which to our best knowledge has not been reported with any fluorescence probe so far. Remarkable also is the dynamic range of this effect, which extends with the same regularity in the direction of both negative and positive charges.

The results of our experiments suggest that the flavone probes F and F2 have to reside embedded rather deeply in the bilayer below the lipid-water charged interface. The relatively low-polar and electrostatically neutral flavone moiety should be located in low-polar surroundings and this location should not be changed dramatically by electrostatic interaction of the F2 positively charged amine with the phosphate groups of phospholipids. The glycerol skeleton and the area of carbonyl groups are known to be the most rigid elements of the bilayer [4,5], and probably the binding occurs at these areas. The observation of red-edge effect of significant magnitude is an argument in favor of this possible location.

According to positions of fluorescence maxima and the ratios of their intensities, both probes F and F2 are located not only in low-polar, but in essentially aprotic environments, with the fluorescence properties between acetonitrile and ethyl acetate (see ref. [26]: the results of detailed studies on solvatochromy of these probes are in preparation). The similarity of spectra with those in low-polar aprotic solvents suggests that hydration water is not the major factor that determines the spectroscopic properties in phospholipid vesicles; it produces only the perturbation effect. The magnitude of this effect can be evidenced from the presented above results of the model studies. Probably the complex formation with only a single water molecule can provide a dramatic change of fluorescence spectra. Thus, in the range of possible perturbation factors that are able to modify the fluorescence properties of flavone probes in low-polar environments, the interaction with hydroxylic cosolvent and water in particular is of primary importance.

The probe differential hydration mechanism can provide the rational explanation of the effects of phospholipid charge. The binding sites for the 3-HF probes in bilayers that possess different charge differ by access to hydration water. Greater probe hydration should provide higher relative emission of N* form. The literature data demonstrating higher hydration of phospholipid ester carbonyl groups by IR and Raman spectroscopy [2,3] are in line with this mechanism and the results presented above.

Thus our findings demonstrate the usefulness of 3-HF derivatives for probing the interfacial properties in biomembrane systems and, in particular, of the effects of hydration and of their coupling with the sign and density of charges at the lipid-solvent interface and of the surface electrostatic potential. The efforts are currently in progress to synthesize phospholipid and steroid derivatives of probe F.

REFERENCES

1. M. Langner and K. Kubica (1999) *Chem. Phys. Lipids* **101**, 3–35.
2. M. Pansenkiewicz-Gierula, Y. Takaoka, H. Miyagawa, K. Kitamura, and A. Kusumi (1997) *J. Phys. Chem.* **101**, 3677–3691.
3. W. Hübner and A. Blume (1998) *Chem. Phys. Lipids* **96**, 99–123.
4. S. J. Marrink., D. P. Tieleman, A. R. von Guuren, and H. J. C. Berendsen (1996) *Faraday Disc.* **103**, 191–201.
5. D. P. Tieleman, S. J. Marrink, and H. J. C. Berendsen (1997) *Biochim. Biophys. Acta* **1331**, 235–270.
6. L. M. Loew (1982) *J. Biochem. Biophys. Meth.* **6**, 243–260.
7. R. J. Clarke, A. Zounim, and J. F. Holzwarth (1995) *Biophys. J.* **68**, 1406–1415.
8. R. J. Clarke (1997) *Biochim. Biophys. Acta* **1327**, 269–278.
9. C. Zheng and G. Vanderkooi (1992) *Biophys. J.* **63**, 935–941.
10. K. Gawrisch, D. Ruston, J. Zimmerberg, V. A. Parsegian, R. P. Rand, and N. Fuller (1992) *Biophys. J.* **61**, 1213–1223.
11. W. Shinoda, M. Shimizu, and S. Okazaki (1998) *J. Phys. Chem. B.* **102**, 6647–6654.
12. J. Slavik (1982) *Biochim. Biophys. Acta* **694**, 1–25.
13. K. Kachel, E. Asuncion-Punzalan, and E. London (1998) *Biochim. Biophys. Acta* **1374**, 63–76.
14. R. B. Campbell, S. V. Balasubramanian, and R. M. Straubinger (2001) *Biochim. Biophys. Acta* **1512**, 27–39.
15. T. Parasassi, G. De Stasio, G. Ravagnan, R. M. Rusch, and E. Gratton (1991) *Biophys. J.* **60**, 179–189.
16. E. K. Krasnowska, L. A. Bagatolli, E. Gratton, and T. Parasassi (2001) *Biochim. Biophys. Acta* **1511**, 330–340.
17. J.-H. Alakoskela, and P. K. J. Kinnunen (2001) *Biophys. J.* **80**, 294–304.
18. C. D. Stubbs, C. Ho, and S. J. Slater (1995) *J. Fluoresc.* **5**, 19–28.
19. O. P. Bondar, V. G. Pivovarenko, and E. S. Rowe (1998) *Biochim. Biophys. Acta* **1369**, 119–130.
20. S. M. Dennison, J. Guharay, and P. K. Sengupta (1999) *Spectrochim. Acta*, **A 55**, 1127–1132.
21. P. K. Sengupta and M. Kasha (1979) *Chem. Phys. Lett.* **68**, 382–388.
22. P. -T. Chou, M. L. Martinez, and J.-H. Clements (1993) *J. Phys. Chem.* **97**, 2618–2622.
23. T. C. Swinney and D. F. Killey (1993) *J. Phys. Chem.* **99**, 211–221.
24. S. M. Ormson, R. G. Brown, F. Vollmer, and W. Rettig (1994) *J. Photochem. Phtobiol. A. Chem.* **81**, 65–72.
25. D. Lleres, E. Dauty, J.-P. Behr, Y. Mély, and G. Duportail (2001) *Chem. Phys. Lipid* **111**, 59–71.
26. A. S. Klymchenko, and A. P. Demchenko (2002) *Proc. SPIE-Inst. Soc. Opt. Eng.* (In press.)
27. A. P. Demchenko (1989) *Trends Biochem. Sci.* **10**, 374–377.
28. A. P. Demchenko (1991) in J. R. Lakowicz (Ed.), *Topics in Fluorescence Spectroscopy* vol. 3, Plenum Press, New York, pp. 61–111.
29. A. P. Demchenko (2002) *Luminescence* **17**, 19–42.
30. A. P. Demchenko and N. V. Shcherbatska (1985) *Biophys. Chem.* **22**, 131–143.
31. D. M. Gakamsky, A. P. Demchenko, N. Nemkovich, A. N. Rubinov, V. I. Tomin, and N. V. Shcherbatska (1992) *Biophys. Chem.* **42**, 49–61.